AD_____

Award Number: DAMD17-03-1-0070

TITLE: Role of Nitric Oxide as a Chemosensitizer in Prostate Cancer

PRINCIPAL INVESTIGATOR: Charles H. Graham, Ph.D.

CONTRACTING ORGANIZATION: Queen's University
Kingston, Ontario
Canada K7L-3N6

REPORT DATE: February 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2004	Annual (1 Feb 2003 - 31 Jan 2004)

4. TITLE AND SUBTITLE
Role of Nitric Oxide as a Chemosensitizer in Prostate Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0070

6. AUTHOR(S)
Charles H. Graham, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Queen's University
Kingston, Ontario
Canada K7L 3N6

E-Mail: grahamc@post.queensu.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Most patients who die of prostate cancer do so because of therapy failure. Regional hypoxia is a prevalent feature of prostate carcinomas and hypoxia contributes to chemoresistance in various types of cancer. Thanks to the funding provided by the US Army Medical Research and Material Command (Award no. DAMD17-03-1-0070), we have shown that hypoxia increases resistance to more than one class of chemotherapeutic agents in human and rodent prostate cancer cells. Specifically, our studies reveal that incubation of human (PC-3 and DU-145) as well as mouse (TRAMP-C2) prostatic adenocarcinoma cells under hypoxia (0.5%O_2) significantly increases their resistance to chemotherapeutic drugs that exert their cytotoxic effects via different mechanisms, i.e. doxorubicin and paclitaxel. Moreover, we have also demonstrated that the effect of hypoxia on chemoresistance of prostate cancer cells can be prevented by very low concentrations of nitric oxide mimetic agents (Task 1). As initially proposed in the Statement of Work, we were able to accomplish these objectives within the first 12 months of funding. These findings suggest that it may be feasible to use NO mimetics as adjuvants to chemotherapy in prostate cancer patients.

14. SUBJECT TERMS
Drug resistance, hypoxia, nitric oxide, prostate cancer, chemotherapy, chemosensitivity

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

A large proportion of patients with prostate cancer succumb to this disease because the current gold standard therapies (surgery and radiation) often fail to completely eradicate the tumour. Furthermore, advanced tumours eventually become refractory to hormonal therapy and develop high levels of resistance to a variety of chemotherapeutic agents[1]. Previous studies revealed that a lack of oxygen (hypoxia) is a significant contributor to chemoresistance in various non-prostate cancers[2-8]. While it was known that regional hypoxia is a prevalent feature of prostate carcinomas, a causal relationship between hypoxia and chemoresistance in prostate cancer had not been established. Thus, an important objective of our research funded by the U.S. Army Medical Research and Materiel Command was to determine whether hypoxia contributes to chemoresistance in prostate cancer cells. Work in our laboratory demonstrated that a key mechanism by which hypoxia contributes to the development of chemoresistance in human breast carcinoma and mouse melanoma cells is by interfering with endogenous production of nitric oxide (NO) and subsequent signalling by this molecule[9]. Therefore, another component of our proposal is to determine whether hypoxia-induced chemoresistance in prostate cancer cells is due to a decrease in NO production and whether NO mimetic agents can be used to chemosensitize hypoxic prostate cancer cells.

BODY

Task 1. To determine whether hypoxia and reduced NO levels increase drug resistance in prostate cancer cells (Months 1-24):

Assess the effect of hypoxia and low NO levels on chemoresistance using a panel of human and rodent prostate carcinoma cells treated with various classes of chemotherapeutic agents (Months 1-12).

To date, we have demonstrated that hypoxia indeed increases the survival of various prostate cancer cell lines following exposure to different classes of chemotherapeutic agents. Specifically, our results using colony-formation assays reveal that, compared with culture under standard (20% oxygen) conditions, pre-exposure of human PC-3 and DU-145 prostate adenocarcinoma cells, as well as mouse TRAMP-C2 cells, to hypoxia (0.5% oxygen) for 24 hours increases their survival following a one-hour exposure to doxorubicin[10] and paclitaxel (see attached published *J. Urol.* paper + Keystone Symposium abstract + Fig. 1). In the *J. Urol.* study we also provided evidence that the effect of hypoxia on prostate cancer cell chemoresistance may not be due to major alterations in the cell cycle.

In the clinical setting, small differences in the size of the tumour cell surviving fraction following administration of chemotherapeutic agents can determine therapy success (or failure). While the colony-formation assay is a true measure of tumour cell resistance to chemotherapeutic agents, as only the surviving fraction gives rise to colonies, this is a labour-intensive and time-consuming approach. Depending on the cell type, colonies may take up to four weeks to form after drug administration. Therefore, over the last year we have adapted the MTT (3,-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide) cell viability assay to address some of the research questions in our proposal. In this assay, the yellow MTT tetrazolium salt is converted into a dark formazan product by the mitochondria of live cells. The resulting formazan is then solubilized and quantified spectrophotometrically by reading the absorbance at 590 nm. Unlike the colony-formation assay, the MTT assay yields results faster and, since it is performed in a

96-well tissue culture plate, it allows us to examine the effect of multiple culture conditions on cell survival in a single experiment. As shown in Fig. 2, using the MTT assay we also have been able to show that hypoxia increases the survival of DU-145 cells exposed to doxorubicin.

Conduct assays to determine whether NO mimetics can prevent the effect of hypoxia and NOS inhibition on drug resistance in prostate cancer cells (Months 12-24).

In our published paper, as well as in more recent studies, we also demonstrated that co-incubation of human PC-3 and mouse TRAMP-C2 prostate cancer cells with the NO mimetic drug nitroglycerine (glyceryl trinitrate, GTN; 0.1 nM – 1 μM) inhibited the hypoxia-induced resistance to doxorubicin[10] (see attached: *J. Urol.* paper; Frederiksen *et al.* Kestone Symposium abstract; and Fig. 1). For GTN to function as a NO mimetic, it must first be biotransformed by a variety of enzymes in the cell, including cytochrome P450 enzymes and glutathione-S-transferase. We have now extended these findings to show that isosorbide dinitrate (ISDN), a NO mimetic drug that releases NO via a mechanism of biotransformation different from that of GTN, also chemosensitizes prostate cancer cells exposed to hypoxia (Fig. 3). Our previous findings revealed that diethylenetriamine NO adduct (DETA/NO), a NO mimetic that does not require biotransformation, is capable of chemosensitizing human MDA-MB-231 breast carcinoma cells[9]. These findings suggest that it may be feasible to use different classes of NO mimetics as adjuvants to chemotherapy in prostate cancer patients.

Together with our previous findings, the results of our recent experiments provide evidence that an important mechanism by which hypoxia induces chemoresistance in tumour cells involves the inhibition of endogenous NO production. These processes are likely linked because the presence of O_2 is essential for the generation of NO by the enzyme NOS[11]. As indicated below, an objective of our research program is to determine whether hypoxia inhibits NO production in prostate cancer cells.

We are currently conducting studies to determine whether pharmacological inhibition of endogenous NO synthesis, using NO synthase inhibitors such as N^G-monomethyl-L-arginine (L-NMMA), leads to increased drug resistance in prostate cancer cells. This task is to be accomplished within the second year of funding. While in previous studies we demonstrated that inhibition of endogenous NO production increases chemoresistance in breast cancer cells[9], it is important to determine whether this is also the case for prostate cancer cells.

Task 2. To determine whether hypoxia induces drug resistance in prostate cancer by inhibiting NO production (Months 12-24):

Measure L-citrulline production in prostate cancer cells (Months 12-24).
Measure nitrates and nitrites produced in prostate cancer cells cultured under hypoxia (Months 12-24).

These studies are to be completed within the second year of funding. However, we have conducted initial studies using the Griess reagent assay to determine whether the production of nitrites and nitrates, as markers for NO production, is decreased in PC-3 prostate cancer cells exposed to hypoxia. Results so far have yielded inconclusive data because the levels of nitrites and nitrates produced by these cells are very low and comparable to those found in the culture medium alone (Fig. 4). To address this issue, we will test whether another prostate cancer cell

line, such as DU-145 or LnCap, produces more NO and is therefore more suitable for these studies. As an alternative and complementary approach, we will measure the effect of hypoxia on cyclic guanosine monophosphate (cGMP) levels in PC-3 and DU-145 cells. cGMP is a second messeger responsible for many of the biological effects of NO and is produced following activation of the enzyme soluble guanylyl cyclase (sGC) by NO. If hypoxia decreases NO production, cGMP levels should also In a previous study, we showed that hypoxia decreases the production of cGMP in human MDA-MB-231 breast cancer cells[12].

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that hypoxia contributes to resistance to chemotherapeutic agents in prostate cancer cells.
- Provided evidence that the effect of hypoxia on prostate cancer cell chemoresistance can be attenuated by very low concentrations of NO mimetic agents such as nitroglycerin and ISDN.
- Adapted the MTT cell viability assay to our system. This will allow us to dissect the mechanism by which NO mimetics chemosensitize hypoxic prostate cancer cells.

REPORTABLE OUTCOMES

- Paper published last fall in the *Journal of Urology* (enclosed). Another manuscript is in preparation in which we will publish the broad effect of hypoxia on resistance of various human prostate cancer cells to different classes of chemotherapeutic agents.
- Abstract to be presented at the Keystone Symposium on Hypoxia to be held in March 2004 at Steamboat Springs, CO.
- Based on this grant we were awarded a research contract by Cellegy Pharmaceuticals to test the chemosensitizing effect of nitroglycerin in a mouse model of prostate cancer.

CONCLUSIONS

- Our findings indicate that hypoxia contributes to the development of drug resistance in prostate cancer cells and that NO may play an important role in the regulation of chemosensitivity.
- Results suggest that administration of GTN may provide a means of chemosensitizing prostatic carcinomas.
- The outcomes of our studies may lead to the development of novel approaches to increase the efficacy of current chemotherapeutic modalities. Such approaches may result in greater survival benefit and improvement in the quality of life of prostate cancer patients.

REFERENCES

1. Eisenberger, M.A., Simon, R., O'Dwyer, P.J., Wittes, R.E., and Friedman, M.A. (1985) A reevaluation of nonhormonal cytotoxic chemotherapy in the treatment of prostatic carcinoma. *J. Clin. Oncol.* **3**:827-841.

2. Kalra, R., Jones, A.M., Kirk, J., Adams, G.E., and Stratford, I.J. (1993) The effect of hypoxia on acquired drug resistance and response to epidermal growth factor in Chinese hamster lung fibroblasts and human breast-cancer cells in vitro. *Int. J. Cancer* **54**:650-655.

3. Smith, E., Stratford, I.J., and Adams, G.E. (1980) Cytotoxicity of adriamycin on aerobic and hypoxic chinese hamster V79 cells in vitro. *Br. J. Cancer* **42**:568-573.

4. Rice, G.C., Ling, V., and Schimke, R.T. (1987) Frequencies of independent and simultaneous selection of Chinese hamster cells for methotrexate and doxorubicin (adriamycin) resistance. *Proc. Natl. Acad. Sci. U S A* **84**:9261-9264.

5. Wilson, R.E., Keng, P.C., and Sutherland, R.M. (1989) Drug resistance in Chinese hamster ovary cells during recovery from severe hypoxia. *J. Natl. Cancer Inst.* **81**:1235-1240.

6. Sanna, K., Rofstad, E.K. (1994) Hypoxia-induced resistance to doxorubicin and methotrexate in human melanoma cell lines in vitro. *Int. J. Cancer* **58**:258-262.

7. Luk, C.K., Veinot-Drebot, L., Tjan, E., and Tannock, I.F. (1990) Effect of transient hypoxia on sensitivity to doxorubicin in human and murine cell lines. *J. Natl. Cancer Inst.* **82**:684-692.

8. Liang, B.C. (1996) Effects of hypoxia on drug resistance phenotype and genotype in human glioma cell lines. *J. Neurooncol.* **29**:149-155.

9. Matthews, N.E., Adams, M.A., Maxwell, L.R., Gofton, T.E., and Graham, C.H. (2001) Nitric oxide-mediated regulation of chemosensitivity in cancer cells. *J. Natl. Cancer Inst.* **93**:1879-1885.

10. Frederiksen, L.J., Siemens, D.R., Heaton, J.P., Maxwell, L.R., Adams, M.A., and Graham, C.H. (2003) Hypoxia induced resistance to doxorubicin in prostate cancer cells is inhibited by low concentrations of glyceryl trinitrate. *J. Urol.* **170**:1003-1007.

11. McCormick, C.C., Li, W.P., and Calero, M. (2000) Oxygen tension limits nitric oxide synthesis by activated macrophages. *Biochem. J.* **350**:709-716.

12. Postovit, L.M., Adams, M.A., Lash, G.E., Heaton, J.P., and Graham, C.H. (2002) Oxygen-mediated regulation of tumour cell invasiveness: involvement of a nitric oxide signalling pathway. *J. Biol. Chem.* **277**:35730-35737.

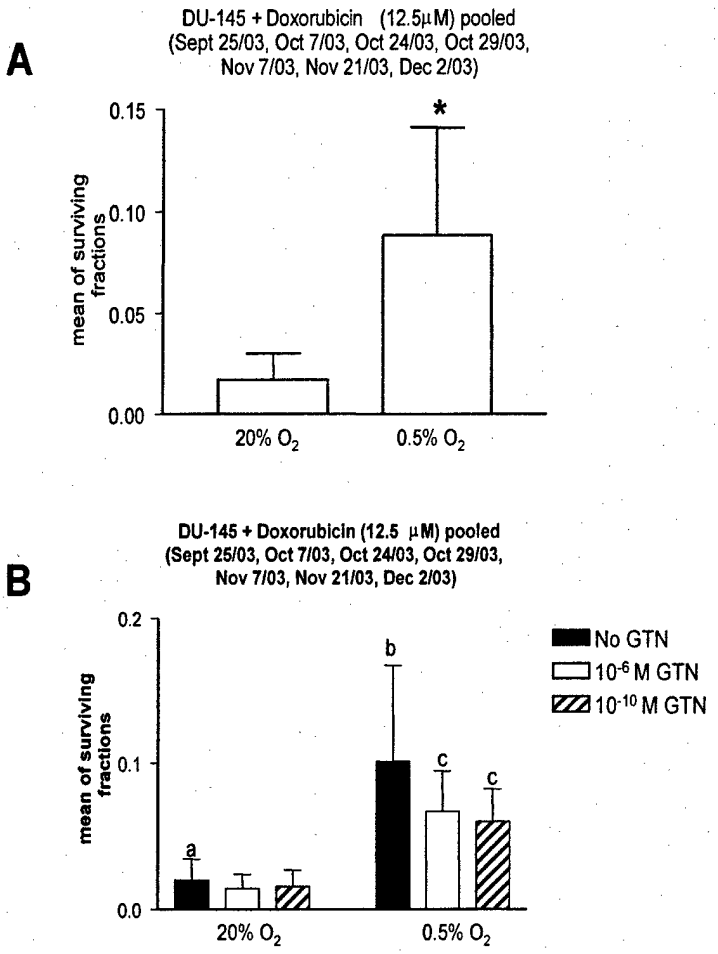

Figure 1. Effect of hypoxia and glyceryl trinitrate on the survival of DU-145 cells following a 1-h exposure to doxorubicin (12.5 μM). Survival was assessed by colony formation assay. Asterisk in A represents significant difference ($P < 0.05$; Student's t-test). In B, a and c are statistically significantly different from b ($P < 0.05$; one-way ANOVA followed by Fisher's test for statistical significance).

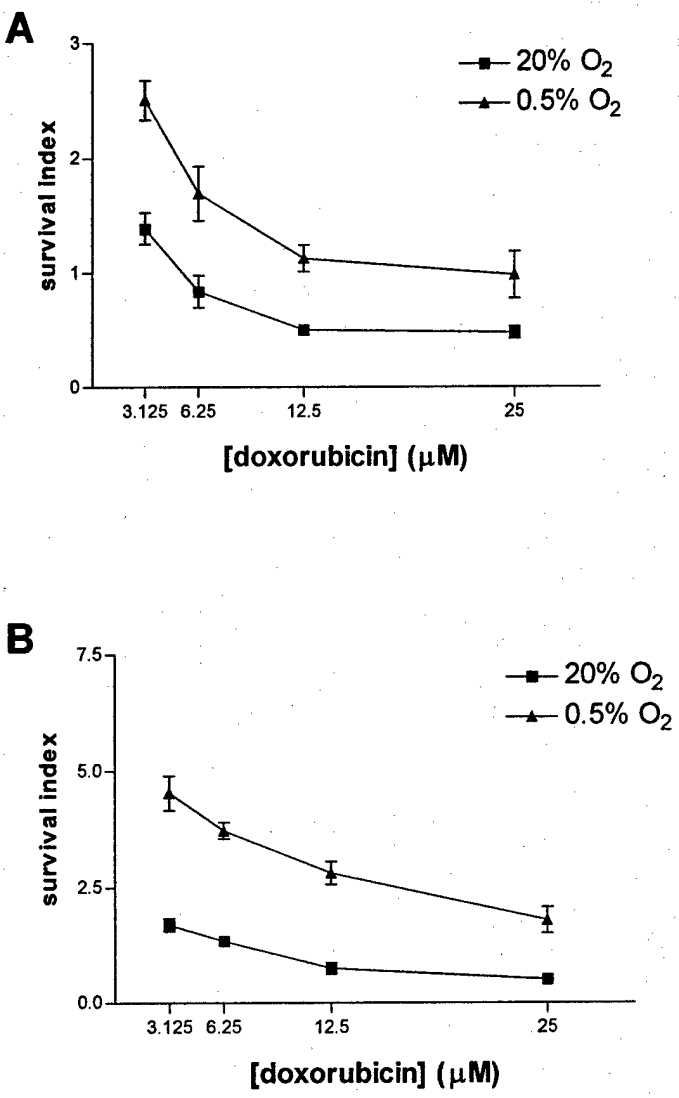

Figure 2. Effect of oxygen concentration on the survival of DU-145 cells following doxorubicin exposure as determined by the MTT assay. In A (top) survival was determined 8 days following a 1 h exposure to doxorubicin whereas in B survival was assessed 10 days after doxorubicin exposure.

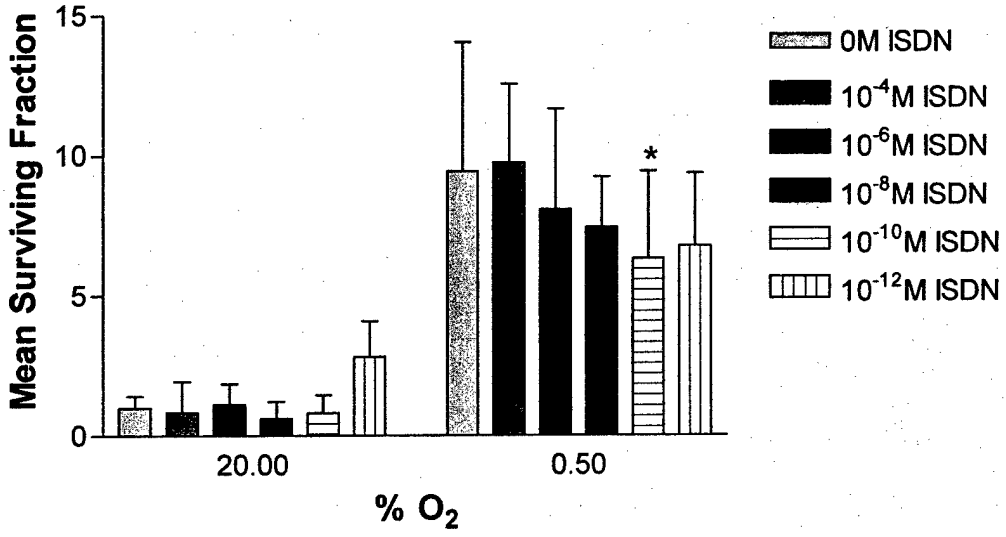

Figure 3. Effect of oxygen and isosorbide dinitrate (ISDN) on the survival of DU-145 prostate cancer cells following a 1-h exposure to doxorubicin (25 μM). A statistically significant attenuation of the hypoxia-induced resistance to doxorubicin was observed when cells were incubated in 0.5% O_2 in the presence of 10^{-10}M doxorubicin ($P < 0.05$; one-way ANOVA followed by Fisher's test for statistical significance). Interestingly, higher concentrations of ISDN were unable to chemosensitize the prostate cancer cells. This is a 'low concentration' effect of NO, likely involving the activation of soluble guanylyl cyclase and cGMP

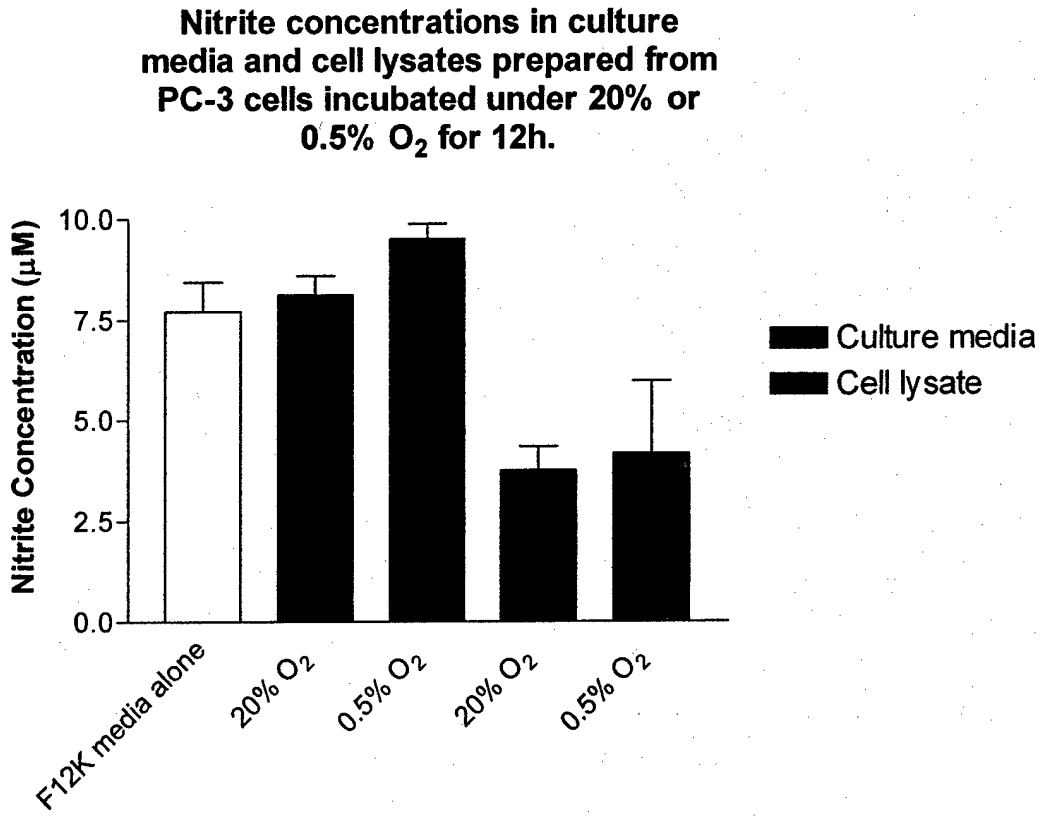

Figure 4. Nitrite concentration in the culture media and lysates of PC-3 cells incubated in 20% or 0.5% O_2 for 12 h. As indicated in the text, nitrite concentrations in both the conditioned media (black bars) and cell lysates (grey bars) were very low and similar to those present in the fresh culture medium (white bar).

Abstract presented at Keystone Symposium on Hypoxia, Steamboat Springs, Colorado. March 25-30, 2004.

Multi-drug resistance in prostate cancer is induced by hypoxia

Lisa J. Frederiksen[1], D. Robert Siemens[1,2], Jeremy P. Heaton[2,3], Michael A. Adams[2,3] and Charles H. Graham[1,3], Department of Anatomy and Cell Biology[1], Urology[2], and Pharmacology and Toxicology[3], Queen's University, Kingston, Ontario, Canada, K7L 3N6

Low tumour oxygenation (hypoxia) correlates with increased metastasis and resistance to radiotherapy and chemotherapy. Hypoxia has been shown to be associated with human prostate cancer, and prostate cancers are often highly resistant to chemotherapy. Recently, we have demonstrated that hypoxia induces resistance to doxorubicin in both human PC-3 and mouse TRAMP-C2 prostate cancer cells. We hypothesized that hypoxia induces tumour cell resistance to drugs that exert their cytotoxic effects via different pathways. PC-3, DU-145, and TRAMP-C2 prostatic adenocarcinoma cells were used for chemosensitivity assays using drugs of different mechanisms (doxorubicin and paclitaxel). These cell lines were incubated in 20% O_2 or 0.5% O_2 for 24 hours followed by a one-hour incubation with the chemotherapeutic. Survival was assessed by colony formation assays. Pre-incubation of all three cell lines under hypoxia resulted in increased survival following exposure to paclitaxel. There was an observed increase of 1.8 fold for PC-3 cells ($P < 0.047$), 1.3 fold for DU-145 cell ($P < 0.005$) and 1.6 fold for TRAMP-C2 cells ($P < 0.0001$). In confirmation of our previous studies using PC-3 and TRAMP-C2 prostate cancer cells, DU-145 cells exhibited hypoxia-induced resistance (5.4 fold) to doxorubicin ($P < 0.0005$). These findings indicate that hypoxia interferes with the cell killing effects of various classes of chemotherapeutic agents. Intervention in one or more steps of the mechanism of hypoxia-induced chemoresistance may provide a novel approach to enhance therapeutic efficacy in prostate cancer patients. (Supported by Canadian Institute of Health Research and United States Army Medical Research and Materiel Command DAMD17 03-1-0070)

Lisa Frederiksen
Telephone Number: 613-533-6000 ext. 74198
Meeting Code: D3

0022-5347/03/1703-1003/0
THE JOURNAL OF UROLOGY®

Vol. 170, 1003–1007, September 2003
Printed in U.S.A.
DOI: 10.1097/01.ju.0000081126.71235.e0

HYPOXIA INDUCED RESISTANCE TO DOXORUBICIN IN PROSTATE CANCER CELLS IS INHIBITED BY LOW CONCENTRATIONS OF GLYCERYL TRINITRATE

LISA J. FREDERIKSEN, D. ROBERT SIEMENS, JEREMY P. HEATON, LORI R. MAXWELL,* MICHAEL A. ADAMS AND CHARLES H. GRAHAM*,†

From the Departments of Anatomy and Cell Biology (LJF, LRM, CHG), Urology (DRS, JPH, MAA) and Pharmacology and Toxicology (JPH, MAA, CHG), Queen's University, Kingston, Ontario, Canada

ABSTRACT

Purpose: Tumor hypoxia has been correlated with metastasis and resistance to chemotherapy. Hypoxia is also associated with human prostate cancers, which are highly resistant to chemotherapy. We hypothesized that hypoxia contributes to chemoresistance in prostate cancer cells and this hypoxia induced chemoresistance can be inhibited by low concentrations of nitric oxide (NO) mimetics.

Materials and Methods: Human PC-3 and mouse TRAMP-C2 prostatic adenocarcinoma cells were incubated in 20% or 0.5% O_2 for 12 hours with or without glyceryl trinitrate (GTN) (0.1 nM). This treatment was followed by a 1-hour incubation with doxorubicin and survival was assessed by clonogenic assays. Western blot analysis was used to measure NO synthase levels. The effect of hypoxia and GTN on cell cycle distribution was determined by flow cytometry.

Results: Hypoxic pre-incubation of the 2 cell lines resulted in increased survival following exposure to doxorubicin. Co-incubation of PC-3 and TRAMP-C2 cells with GTN (0.1 nM) inhibited the hypoxia induced resistance to doxorubicin. Each cell line expressed all 3 NO synthase isoforms at levels that were not significantly affected by O_2 concentrations. Cell cycle analysis revealed that there was no significant difference in the distribution of PC-3 cells at each stage of the cycle. However, incubation under hypoxia resulted in a small decrease in the number of TRAMP-C2 cells in S-phase.

Conclusions: These findings indicate that NO may have an important role in the regulation of chemosensitivity in prostate cancer cells. Furthermore, the results suggest that GTN administration may represent a means of chemosensitizing prostatic carcinomas.

KEY WORDS: prostate, adenocarcinoma, nitric oxide, anoxia, drug resistance

Despite extensive research, there remain few treatment options for men with prostatic carcinomas who progress to a hormone refractory state with patients having a median survival of approximately 18 months.[1] Chemotherapeutic regimens for prostate cancer have not demonstrated a definitive survival benefit since the median survival for patients treated with chemotherapy is not significantly greater than for those who receive conventional supportive care.[1,2] In addition, advanced prostatic carcinomas often show intrinsically high levels of resistance to various anticancer drugs.[3] Many mechanisms of drug resistance in prostate cancer have been shown to be associated with mutations or inactivation of the p53 suppressor gene and over expression of bcl-2.[3]

Hypoxia occurs in most solid tumors and it has been shown to be an independent prognostic indicator of poor clinical outcome for patients with various cancers.[4] In 2001 Movsas et al reported the first evidence of seriously altered intratumoral O_2 levels in patients with prostate cancer.[5] Their findings revealed that tissue O_2 levels in prostate cancer lesions were substantially lower than in normal tissue,[5,6] and the extent of hypoxia in prostatic tumors correlated with a negative clinical outcome.[6]

Tumor hypoxia has also been shown to correlate with increased tumor invasion and metastasis[7,8] as well as resistance to certain chemotherapeutic agents.[4] This resistance to anticancer drugs has been attributed in part to a lack of O_2 available for drug action, an induction of glucose regulated proteins and/or oxygen regulated proteins, DNA over replication, increased genetic instability, the antiproliferative effects of hypoxia[9] and most recently to an increase in the multidrug resistance transporter P-glycoprotein.[10] However, the nature of this resistance is still not well understood. Studies from our laboratory have revealed that transient exposure to hypoxia induces a reversible drug resistance phenotype in human breast carcinoma and mouse melanoma cells.[11] Moreover, there is also evidence that the suppression of endogenous nitric oxide (NO) production is an essential aspect of the mechanism underlying hypoxia induced chemoresistance.[11] NO is known to have many physiological functions, including acting as a potent vasodilator, a neurotransmitter and a regulator of gene expression.[12] There is also evidence that NO inhibits hypoxia inducible factor-1 (HIF-1) DNA binding activity[13] and HIF-1α accumulation[14] in hypoxic cells, further demonstrating an association between NO and hypoxia. HIF-1 is a transcription factor involved in the oxygen dependent regulation of many genes, including vascular endothelial growth factor[15] and erythropoietin.[16] In more recent studies we observed that exposure of human breast carcinoma cells to hypoxia decreased the

Accepted for publication March 28, 2003.
Supported by a grant from the Prostate Cancer Research Foundation of Canada, an Idea Development Award from the United States Department of Defense and Cellegy Pharmaceuticals, Inc.
* Financial interest and/or other relationship with Cellegy Pharmaceuticals.
† Corresponding author and requests for reprints: Department of Anatomy and Cell Biology, Botterell Hall, Room 859, Queen's University, Kingston, Ontario, Canada K7L 3N6 (telephone: 613-533-2852; FAX: 613-533-2566; e-mail: grahamc@post.queensu.ca).

intracellular levels of cyclic guanosine monophosphate,[7] a second messenger generated upon activation of soluble guanylyl cyclase by NO.[17]

In light of the previous studies showing that hypoxia has an important role in the development of drug resistance in various types of cancer, we hypothesized that hypoxia is an important factor in the development of drug resistance in prostate cancer cells. Thus, we determined whether hypoxia increases the resistance of prostate cancer cells to doxorubicin and whether this hypoxia induced drug resistance can be attenuated by low concentrations of glyceryl trinitrate (GTN) (Sabex, Inc., Boucherville, Quebec, Canada), a NO mimetic agent.

MATERIALS AND METHODS

Cells. Human PC-3 prostatic adenocarcinoma cells (American Type Culture Collection, Manassas, Virginia) and mouse TRAMP-C2 prostate tumor cells were used. PC-3 cells were maintained in monolayer culture in Kaighn's modification of Hamm's F12 medium supplemented with 10% fetal bovine serum and TRAMP-C2 cells were maintained in Dulbecco's modified Eagle's medium (Gibco Invitrogen Corp., Burlington, Ontario, Canada) supplemented with 5 μg/ml insulin (Sigma-Aldrich Canada, Ltd., Oakville, Ontario, Canada) and 5% fetal bovine serum.

Culture conditions. For incubation in standard conditions (20% O_2) cells plated in 60 mm culture plates (70% to 80% confluent) were placed in a Sanyo (Esbe Scientific, Markham, Ontario, Canada) CO_2 incubator (5% CO_2 in air at 37C). To establish hypoxic conditions cells were placed in airtight chambers (BellCo Biotechnology, Vineland, New Jersey) that were flushed with a gas mixture of 5% CO_2/95% N_2. Oxygen concentrations within these chambers were maintained for 12 hours at 0.5% using Pro-Ox Model 110 (Biospherix, Redfield, New York) O_2 regulators. To determine whether exposure to a NO mimetic agent during incubation in to hypoxia affected tumor cell survival after exposure to a chemotherapeutic agent, randomly selected culture plates were incubated with a low concentration (0.1 nM) of GTN administered at the beginning of hypoxic exposure.

Exposure to doxorubicin and clonogenic (colony formation) assay. Following exposure of cells to 20% or 0.5% O_2 in the presence or absence of GTN cultures were incubated with doxorubicin (Sigma-Aldrich Canada, Ltd.) (0 to 100 μM) for 1 hour at 20% O_2 in a standard CO_2 incubator. Cultures were then washed with drug-free phosphate buffered saline (PBS), harvested with 0.075% trypsin-ethylene diaminetetraacetic acid in PBS and counted using a hemocytometer. Tumor cell survival following doxorubicin exposure was determined by a clonogenic assay, as previously described.[11] In preliminary studies it was determined that seeding 1,000 PC-3 and 5,000 TRAMP-C2 cells of the doxorubicin treated populations and 100 PC-3 and 500 TRAMP-C2 cells of the nontreated control groups resulted in appropriate numbers of colonies for consistency and ease of counting. After 7 to 14 days colonies were fixed with acetic acid-methanol (1:4) and stained with dilute crystal violet (1:30) prior to being counted.

Western blot analysis. Following incubation cells were lysed in 2% sodium dodecyl sulfate, 10 mM tris HCl (pH 7.5) and 0.15 mM NaCl. The lysates were homogenized, followed by DNA shearing (10 times with a 25⅝ gauge needle), boiling (5 minutes) and centrifugation (15 minutes at 14,000 × gravity). The supernatant was collected and stored at −80C until use. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Resolved proteins were transferred onto an Immobilon-P (Millipore Corp., Bedford, Massachusetts) membrane using a wet transfer apparatus (BioRad Laboratories, Mississauga, Ontario, Canada). The membranes were blocked overnight at 4C in a solution containing 1% PBS-0.01% Tween 20 (PBS-T) and 10% dry milk powder. The blots were subsequently incubated for 2.5 hours with polyclonal rabbit antiserum raised against human NO synthase (NOS) 1 (1:500), NOS 2 (1:400) or NOS 3 (1:800) (Santa Cruz Biotechnologies, Santa Cruz, California), followed by 3, 5-minute washes with PBS-Tween. Membranes were then incubated for 40 minutes with horseradish peroxidase labeled goat antirabbit IgG secondary antibody (Vector Laboratories, Inc., Burlingame, California) (1:10,000 dilution). Following 3 additional 5-minute washes with PBS-T secondary antibodies were detected by enhanced chemiluminescence and exposure onto Kodak Scientific Imaging Film (Perkin-Elmer Life Sciences, Inc., Boston, Massachusetts). The next day the blots were washed in PBS-T for 10 minutes, followed by incubation in a monoclonal antiβ-actin antibody (Sigma-Aldrich Canada, Ltd.) for 1 hour at room temperature (1:10,000 dilution) and then with horseradish peroxidase labeled goat antimouse IgG secondary antibody (BioRad Laboratories, Hercules, California) for 1 hour (1:10,000).

Flow cytometric analysis of cell cycle. Following 12-hour exposure to 20% O_2 and 0.5% O_2 in the presence or absence of 0.1 nM GTN cells were fixed in ethanol and stained with propidium iodide (500 μg/ml) in the presence of RNAse A (Sigma-Aldrich, Canada, Ltd.) (10 mg/ml) at 37C for 30 minutes. DNA content in cells was analyzed with a Coulter Elite Flow Cytometer (Beckman-Coulter Corp., Miami, Florida).

Calculations and statistical analyses. Plating efficiency was calculated according to the number of surviving colonies expressed as a proportion of the total number of cells seeded. Surviving fractions were determined by dividing the plating efficiency of drug treated groups by the plating efficiency of their respective control groups. X-rays of Western blot experiments were scanned and analyzed using the SigmaGel (SPSS, Inc., Chicago, Illinois) densitometry software package. Density values were normalized using β-actin bands and results are expressed as relative to control cells incubated in 20% O_2. All data are presented as the means ± SD. Statistical analyses were performed using the StatView (Abacus Concepts, Inc., Berkley, California) statistical software package. Statistical significance was determined by 1-way ANOVA, followed by Fisher's post hoc analysis. Student's t test was used when only 2 sets of data were compared. All statistical tests were 2-sided and differences were considered statistically significant at $p < 0.05$.

RESULTS

Exposure to hypoxia and drug resistance. Exposure of TRAMP-C2 and PC-3 cells to 0.5% O_2 for 12 hours significantly increased survival following treatment with various concentrations of doxorubicin, in contrast to cells incubated in the standard condition of 20% O_2 (fig. 1). Since the highest levels of hypoxia induced chemoresistance were observed when 12.5 μM doxorubicin were used (up to 12-fold increase for TRAMP-C2 and up to 7.7-fold increase for PC-3 cells), this dose of doxorubicin was used in all subsequent experiments.

Effect of low concentrations of GTN on the induction of hypoxia associated drug resistance. The relative survival of TRAMP-C2 and PC-3 prostate cancer cells following exposure to doxorubicin (12.5 μM) was not affected by a single administration of GTN (0.1 nM) when the cells were incubated in 20% O_2 ($p = 0.88$ and 0.86, respectively, fig. 2). In contrast, a similar treatment with GTN at the onset of the 12-hour hypoxic incubation (0.5% O_2) completely prevented the development of the hypoxia induced drug resistance phenotype in TRAMP-C2 and PC-3 prostate cancer cells (each $p < 0.0001$).

Expression of NOS enzymes by PC-3 and TRAMP-C2 cells. Western blot analysis showed that all 3 NOS isoforms were present in the 2 prostate cancer cell lines (fig. 3). Incubation in different concentrations of O_2 did not significantly affect the levels of any NOS isoforms detected in either cell lines.

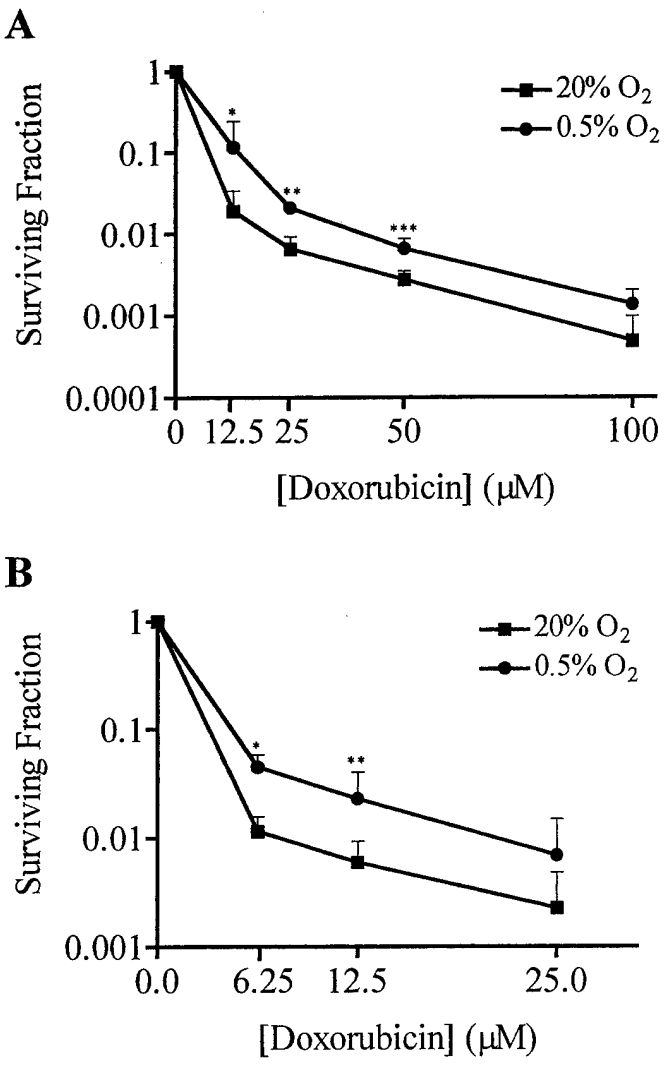

FIG. 1. Mean effect ± SD of O_2 concentration on survival of 6 preparations of PC-3 cells (*A*) and 12 preparations of TRAMP-C2 cells (*B*) exposed to various doxorubicin concentrations. Statistically higher survival levels were observed in cultures incubated in 0.5% (circles) vs 20% (squares) O_2 for 12 hours and subsequently exposed to doxorubicin for 1 hour at concentrations indicated. Single asterisk indicates $p < 0.0001$. Double asterisks indicate $p < 0.005$. Triple asterisks indicate $p < 0.05$.

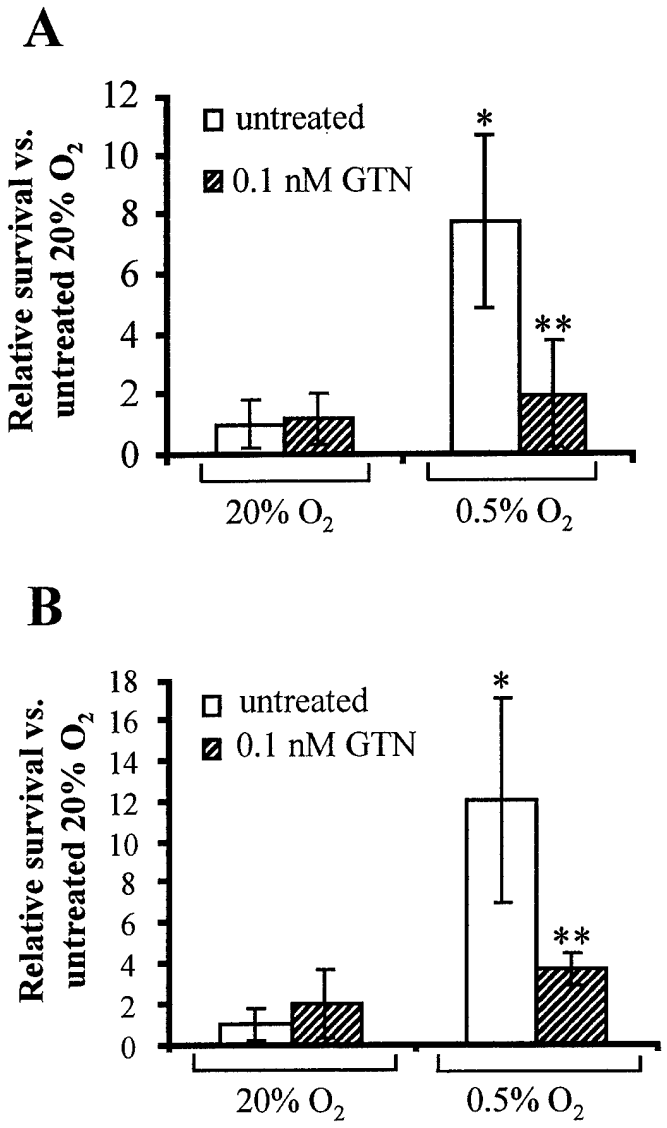

FIG. 2. Mean effect ± SD of single 0.1 nM GTN dose in 12 preparations each of human PC-3 (*A*) and mouse TRAMP-C2 (*B*) cells prevented acquisition of hypoxia induced resistance to 12.5 μM doxorubicin for 12-hour incubation in hypoxia. Single asterisk indicates statistically significant difference in survival compared with cells incubated in 20% O_2 at 12.5 μM doxorubicin ($p < 0.0001$). Double asterisks indicate statistically significant difference in survival compared with cells incubated in 0.5% O_2 alone ($p < 0.0001$).

However, there was an observed trend toward increased NOS 2 expression in PC-3 cells incubated in 0.5% versus 20% O_2 ($p = 0.056$).

Effect of 12-hour hypoxic incubation on cell cycle. Flow cytometric analysis of PC-3 cells revealed no statistically significant differences in the proportions of cells at each stage of the cycle in any treatment populations (see table). A slight but significant decrease in the proportion of cells in S-phase was observed in TRAMP-C2 cells incubated in 0.5% O_2 versus 20% O_2 ($p < 0.04$, see table). Analysis was performed 3 times in duplicate.

DISCUSSION

These novel findings provide evidence of hypoxia induced chemoresistance in prostate cancer cells and that administration of low concentrations of a NO mimetic agent can prevent this form of acquired drug resistance. Specifically the results reveal that hypoxia increases resistance to doxorubicin in androgen independent human and mouse prostate tumor cells, and this hypoxia induced chemoresistance can be prevented by nanomolar concentrations of the NO mimetic GTN.

In a previous study we have provided evidence that the mechanism by which hypoxia induces chemoresistance in tumor cells involves the inhibition of endogenous NO production.[11] These processes are likely associated because the presence of O_2 is essential for the generation of NO by the enzyme NOS.[18] In the current study PC-3 and TRAMP-C2 cells were found to express all 3 NOS isoforms and, therefore, have the capacity to generate NO when all necessary cofactors are present, ie when O_2 is available in sufficient quantities. Although there was a trend toward increased NOS 2 levels in PC-3 cells exposed to 0.5% O_2 for 12 hours, it is unlikely that this increase in NOS translated into elevated NO production because a lack of available O_2 in culture conditions would have limited NO generation by NOS.

Further evidence in support of the concept that hypoxia and decreased NO production are associated with the development of chemoresistance was revealed in the finding that

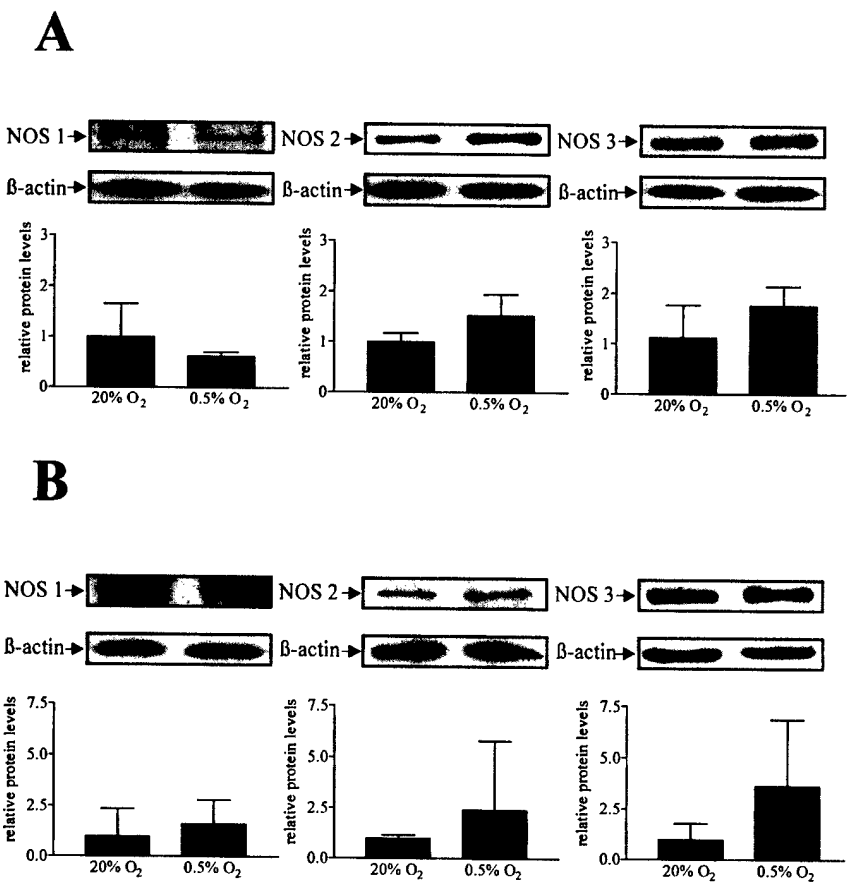

FIG. 3. Western blot analysis of 3 preparations each of human PC-3 (*A*) and mouse TRAMP-C2 (*B*) prostate cancer cells shows NOS isoforms 1 to 3 at 20% and 0.5% O_2. Bars represent mean relative density ± SD.

Cell cycle distribution of PC-3 and TRAMP-C2 cells incubated for 12 hours

Incubation Condition	Mean % Gated ± SD		
	G_0/G_1	S	G_2/M
PC-3 cells:			
20% O_2	61.6 ± 2.4	50.4 ± 3.5	34.9 ± 3.2
0.5% O_2	57.8 ± 3.9	48.7 ± 3.9	36.4 ± 1.9
20% O_2 + 0.1 nM GTN	60.2 ± 2.9	51.3 ± 3.9	36.2 ± 1.7
0.5% O_2 + 0.1 nM GTN	58.1 ± 7.7	46.1 ± 4.4	38.6 ± 7.1
TRAMP-C2 cells:			
20% O_2	52.0 ± 8.5	56.8 ± 7.1	43.3 ± 7.6
0.5% O_2	60.8 ± 3.4	49.3 ± 2.5*	34.4 ± 4.0
20% O_2 + 0.1 nM GTN	56.0 ± 5.7	53.0 ± 4.1	40.0 ± 6.4
0.5% O_2 + 0.1 nM GTN	56.1 ± 14.4	47.0 ± 3.0*	34.5 ± 6.6

* Statistically significantly different vs 20% O_2 ($p < 0.05$).

administration of low concentrations of GTN increased chemosensitivity only in cells pre-exposed to hypoxia (0.5% O_2) and not in cells maintained under high levels of O_2 (ie not in cells with normal NO production capacity). This selective action of NO mimetics to hypoxic cells is clinically important because any therapeutic approach to chemosensitize tumors should target hypoxic, drug resistant cell populations.

GTN was originally described as a potent vasodilator used to treat angina pectoris and congestive heart failure. It is considered a prodrug because it requires biotransformation to its active metabolite NO, mediated by several proteins, such as glutathione S-transferases and cytochrome P450-reduced nicotinamide adenine dinucleotide phosphate cytochrome P450 reductase systems, before it can initiate a pharmacological effect.[19] In addition, there is evidence that the biotransformation of GTN is increased under hypoxia.[20] Therefore, GTN represents an important and useful agent for selectively targeting hypoxic cells.

In tissue culture 20% O_2 is equivalent to a partial O_2 pressure of approximately 150 mm Hg and 5% O_2 (35 to 40 mm Hg) is similar to levels present in the venous circulation and other nonhypoxic regions of the body. Concentrations below 0.5% O_2 (less than 10 mm Hg) characterize tissues with compromised blood flow, such as most solid tumors, including prostatic carcinomas.[5] In the current study we compared the effects of incubation in 0.5% vs 20% O_2 on the development of resistance to doxorubicin and on the ability of a low concentration of GTN to inhibit this resistance. While O_2 concentrations corresponding to 20% would be considered high, previous studies in our laboratory have shown that drug treated cells incubated in 5% O_2 or 20% O_2 responded similarly.[11]

It has been proposed that a mechanism of action of chemotherapeutic agents involves the generation of cytotoxic radicals via a process that depends on the presence of O_2.[9] In the current study all incubations with doxorubicin were performed at 20% O_2. This approach ensured that a lack of radical formation resulting from decreased O_2 would not become a confounding variable in explaining the increased chemoresistance observed in cells pre-exposed to hypoxia. In addition, clonogenic assays were maintained in 20% O_2 during colony formation to allow normal proliferative abilities of the cells regardless of previous exposure to low levels of O_2. Exposure of the tumor cells to doxorubicin was also limited to only 1 hour to avoid genetic alterations that could result from long-term drug treatment since the main objective was to determine the effect induced by hypoxic pre-incubation alone.

Another mechanism by which hypoxia could increase chemoresistance is by decreasing tumor cell proliferation since chemotherapeutic agents are known primarily to target rapidly dividing cells.[9] In our study cell cycle analysis revealed

that hypoxia did not affect any phases of the cycle in PC-3 cells and only had a minor but statistically significant effect on the S-phase of TRAMP-C2 cells. Therefore, it is unlikely that the observed effect of hypoxia on chemoresistance was due to alterations in proliferative ability.

CONCLUSIONS

Combined with our previous studies the current results provide evidence that hypoxia is a major contributor to chemoresistance in prostate cancer and a deficiency in NO signaling may be associated with this hypoxia induced chemoresistance. Consequently the administration of drugs that restore NO signaling, such as NO mimetic agents, may be a useful approach to chemosensitize hormone refractory prostate cancer.[7,11]

TRAMP-C2 prostate tumor cells were provided by Dr. Barbara Foster, Shadyside Medical Center, Pittsburgh, Pennsylvania.

REFERENCES

1. Harris, K. A. and Reese, D. M.: Treatment options in hormone-refractory prostate cancer: current and future approaches. Drugs, **61:** 2177, 2001
2. Odrazka, K., Vanasek, J., Vaculikova, M., Stejskal, J. and Filip, S.: The role of chemotherapy in prostate cancer. Minireview. Neoplasma, **47:** 197, 2000
3. DiPaola, R. S. and Aisner, J.: Overcoming bcl-2- and p53-mediated resistance in prostate cancer. Semin Oncol, **26:** 112, 1999
4. Vaupel, P., Kelleher, D. K. and Hockel, M.: Oxygen status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. Semin Oncol, **28:** 29, 2001
5. Movsas, B., Chapman, J. D., Hanlon, A. L., Horwitz, E. M., Pinover, W. H., Greenberg, R. E. et al: Hypoxia in human prostate carcinoma: an Eppendorf PO2 study. Am J Clin Oncol, **24:** 458, 2001
6. Movsas, B., Chapman, J. D., Greenberg, R. E., Hanlon, A. L., Horwitz, E. M., Pinover, W. H. et al: Increasing levels of hypoxia in prostate carcinoma correlate significantly with increasing clinical stage and patient age: an Eppendorf pO(2) study. Cancer, **89:** 2018, 2000
7. Postovit, L. M., Adams, M. A., Lash, G. E., Heaton, J. P. and Graham, C. H.: Oxygen-mediated regulation of tumor cell invasiveness. Involvement of a nitric oxide signaling pathway. J Biol Chem, **277:** 35730, 2002
8. Young, S. D., Marshall, R. S. and Hill, R. P.: Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci USA, **85:** 9533, 1988
9. Teicher, B. A.: Hypoxia and drug resistance. Cancer Metastasis Rev, **13:** 139, 1994
10. Wartenberg, M., Ling, F. C., Muschen, M., Klein, F., Acker, H., Gassmann, M. et al: Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor-1 (HIF-1) and reactive oxygen species. FASEB J, **17:** 503, 2003
11. Matthews, N. E., Adams, M. A., Maxwell, L. R., Gofton, T. E. and Graham, C. H.: Nitric oxide-mediated regulation of chemosensitivity in cancer cells. J Natl Cancer Inst, **93:** 1879, 2001
12. Hanafy, K. A., Krumenacker, J. S. and Murad, F.: NO, nitrotyrosine, and cyclic GMP in signal transduction. Med Sci Monit, **7:** 801, 2001
13. Huang, L. E., Willmore, W. G., Gu, J., Goldberg, M. A. and Bunn, H. F.: Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide. Implications for oxygen sensing and signaling. J Biol Chem, **274:** 9038, 1999
14. Sandau, K. B., Fandrey, J. and Brune, B.: Accumulation of HIF-1 alpha under the influence of nitric oxide. Blood, **97:** 1009, 2001
15. Liu, Y., Christou, H., Morita, T., Laughner, E., Semenza, G. L. and Kourembanas, S.: Carbon monoxide and nitric oxide suppress the hypoxic induction of vascular endothelial growth factor gene via the 5′ enhancer. J Biol Chem, **273:** 15257, 1998
16. Semenza, G. L. and Wang, G. L.: A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol, **12:** 5447, 1992
17. Denninger, J. W. and Marletta, M. A.: Guanylate cyclase and the .NO/cGMP signaling pathway. Biochim Biophys Acta, **1411:** 334, 1999
18. McCormick, C. C., Li, W. P. and Calero, M.: Oxygen tension limits nitric oxide synthesis by activated macrophages. Biochem J, **350:** 709, 2000
19. Chen, Z., Zhang, J. and Stamler, J. S.: Identification of the enzymatic mechanism of nitroglycerin bioactivation. Proc Natl Acad Sci USA, **99:** 8306, 2002
20. Agvald, P., Adding, L. C., Artlich, A., Persson, M. G. and Gustafsson, L. E.: Mechanisms of nitric oxide generation from nitroglycerin and endogenous sources during hypoxia in vivo. Br J Pharmacol, **135:** 373, 2002